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Pathogenesis of Salmonellosis: Salmonella Exotoxins

Annual Progress Report

(12/1/77-9/1/78)

Johnny W. Peterson, Ph.D.

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This annual report summarizes research progress during the first year of the above mentioned research contract. The first section of this report is a manuscript which was presented at the "Fifth International Symposium on Intestinal Microecology" in Columbia, Missouri on June 1, 1978. It has been reviewed and accepted for publication in the American Journal of Clinical Nutrition, and we expect that will appear in the February issue. The manuscript is included here since it contains most of the research data on

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Salmonella exotoxins generated during this period (12/1/77-9/1/78). The manuscript is followed by a summary of additional results which we feel will benefit future research in this area. Two clinical isolates of Salmonella typhimurium were shown to produce two skin permeability factors. One factor was heat stable and rapid in onset while the other was heat labile and elicited maximal induration by 18-24 hrs. The rapid, erythematous PF response could not be prevented by antisera to cholera toxin or Salmonella antisomatic serum, but it could be simulated by high concentrations of LPS from S. typhimurium. The appearance of the delayed PF reaction was indistinguishable from that of purified cholera toxin. Histological comparisons of rabbit skin injected with Salmonella delayed PF and cholera toxin revealed that both toxins resulted in gross edema and infiltration of PMN's after 18 hours. The Salmonella delayed PF was shown to be resistant to a variety of enzymes, sensitive to extremes in pH, and had an isoelectric point of pH 4.8. Unlike Salmonella LPS skin activity, the Salmonella delayed PF was destroyed at 100°C and was neutralized by monospecific cholera antitoxin. The Salmonella delayed PF, which shares antigenic determinants with cholera toxin, appears to be elaborated by living S. typhimurium cells in the rabbit ligated intestine, since rabbits immunized with procholeragenoid were protected against fluid loss from live cell challenge. Finally, production of the rapid PF is a stable genetic trait, while delayed PF production is apparently an unstable characteristic among the salmonellae.

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II. Manuscript
"Evidence of a Role for Permeability Factors in the
Pathogenesis of Salmonellosis"

Evidence of a Role   Permeability Factors in the Pathogenesis

of Salmonellosis

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ABSTRACT

Two clinical isolates of <u>Salmonella typhimurium</u> were shown to produce two skin permeability factors. One factor was heat stable and rapid in onset while the other was heat labile and elicited maximal induration by 18-24 hrs. The rapid, erythematous PF response could not be prevented by antisera to cholera toxin or <u>Salmonella</u> antisomatic serum, but it could be simulated by high concentrations of LPS from <u>S. typhimurium</u>. The appearance of the delayed PF reaction was indistinguishable from that of purified cholera toxin. Histological comparisons of rabbit skin injected with <u>Salmonella</u> delayed PF and cholera toxin revealed that both toxins resulted in gross edema and infiltration of PMN's after 18 hours. The <u>Salmonella</u> delayed PF was shown to be resistant to a variety of enzymes, sensitive to extremes in pH, and had an isoelectric point of pH 4.8. Unlike <u>Salmonella</u> LPS skin activity, the <u>Salmonella</u> delayed PF was destroyed at 100°C and was neutralized by monospecific cholera antitoxin. The <u>Salmonella</u> delayed PF, which shares antigenic determinants with cholera toxin, appears to be elaborated by living <u>S. typhimurium</u> cells in the rabbit ligated intestine, since rabbits immunized with procholeragenoid were protected against fluid loss from live cell challenge. Finally, production of the rapid PF is a stable genetic trait, while delayed PF production is apparently an unstable characteristic among the salmonellae.

The pathogenesis of enteric bacterial infections has been the subject of numerous investigations which have revealed several virulence factors including specialized modes of attachment, mechanisms of selective colonization, and invasive potentials for the epithelial surface. Research has also concentrated on the role of toxic factors released from these enteric bacteria. While it is necessary to isolate and characterize each of the toxins, it should not be assumed that the factors necessarily act independently of the delivery mechanisms. Thus, the pathogenic mechanism of most infections involves an intriguing balance of colonizing and toxic factors.

The pathogenesis of Salmonella mediated intestinal infections requires penetration of the epithelial surface by the bacteria (1). Certainly, the salmonellae are noted for their invasive properties; however, no clear cut reason exists to explain why the severity of infection ranges from a food poisoning type of gastroenteritis without apparent blood stream invasion to a systemic enteric fever exemplified by typhoid fever. Since many of the same serotypes appear to be involved in both extremes of Salmonella infections, it is intriguing to speculate that toxic factors produced by the salmonellae may determine the symptoms presented and the virulence potential of the multitude of Salmonella serotypes.

Two skin permeability factors (PF) have been found in sterile culture filtrates of a clinical isolate of Salmonella typhimurium strain 986 (2). Intradermal injections into rabbit skin revealed one factor which was rapid in onset (rapid PF) and characterized by an erythematous

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response detectable within 1-2 hours post injection. The second factor
was observed after 18 to 24 hours (delayed PF) and elicited a markedly
indurated response accompanied by an erythematous zone coincident
with the induration area. The rapid PF was observed to be heat stable
at 100°C for at least 4 hr, while the delayed PF was heat labile, being
completely destroyed within 30 minutes at 75°C, and within 4 hr at 56°C.
The skin reaction produced by the delayed PF was indistinguishable in
appearance from the permeability reactions of Vibrio cholerae and Escherichia
coli enterotoxins. Unlike the easily detectable heat stable rapid PF,
the heat labile delayed PF tended to be inhibited in crude culture
filtrates; however, it was detected in preparations which had undergone
partial purification by chromatography through Sephadex G-100. In
addition, the delayed PF was observed to elongate Chinese hamster ovary
(CHO) cells in a manner identical to that of cholera toxin (3). Interestingly,
the CHO cell elongation activity as well as the delayed skin induration
response was neutralized by monospecific antisera to cholera toxin but
not by the preimmunization sera.

The present report attempts to further characterize these
permeability factors, particularly the heat labile delayed permeability
factor from Salmonella typhimurium. We hope eventually to determine the
precise role of these toxins in the pathogenesis of salmonellosis and to
compare their mechanisms of action with those of other related enterotoxins.

Materials and Methods

Organisms. Salmonella typhimurium 936 was isolated during a
1974 outbreak of diarrhea in the pediatric ward of John Sealy Hospital,

Galveston, Tex. It was stored in the lyophilized state and reconstituted at weekly intervals as needed. S. typhimurium 2000 was a recent clinical isolate from the same hospital and was stored at 4°C on Trypticase Soy (BBL) agar slants.

Preparation of cultures. The culture medium used most successfully thus far has been Trypticase Soy broth (BBL) plus 1% Yeast Extract (Difco), although cultures grown in Brain Heart Infusion broth (Difco) have also yielded both toxins. Broth cultures were incubated at 37°C with mild shaking for 24 h. Fermenter cultures of the same medium were incubated at 3C or 37°C for 24 hours with 1 liter/minute aeration and 125 RPM agitation.

Preparation of filtrates. Broth cultures were centrifuged at 12,100 xg, and the supernatants were filtered through 0.2 μm sterile Nalgene filter units either before or after concentration of the filtrates. The filtrates were concentrated by dialysis against 20 M Carbowax (Union Carbide) or in an Amicon ultrafiltration unit fitted with a PM 10 membrane. Filtrates were stored at either 4°C or -20°C.

Purification Procedures. Molecular sieve column chromatography with Sephadex G-100 (Pharmacia) was utilized for partial purification and isolation of the Salmonella exotoxins. Two ml fractions were collected from the column which was equilibrated with Tris-HCl buffer, pH 7.5 (4). The column and fractions were maintained at 4°C.

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Diethylaminoethyl-Sephadex (Pharmacia) was rehydrated in distilled water, washed with Tris-HCl buffer, pH 8. A sample of pooled crude culture filtrates was adjusted to pH 8.0 and was added to the washed DEAE. The mixture was stirred for 1 hr and then filtered using Whatman #1 filter paper. The DEAE was washed with the same Tris buffer until optical density readings (280 nm) of the washes approached zero. For elution of toxin bound to the DEAE, the DEAE was suspended in 0.1 M potassium phosphate buffer, pH 6.0. After stirring for one hour at room temperature, the eluate was filter sterilized and stored at 4°C. The eluate was chromatographed on Sephadex G-100 and the column fractions were assayed for skin permeability activity.

Cholera Toxin. Cholera toxin was purified from fermenter cultures of Vibrio cholerae 569B according to methods published previously (5,6). Procholeragenoid was prepared as described by Finkelstein (7).

Skin testing. Adult New Zealand albino rabbits were shaved, and the remaining hair was removed with a depilatory cream prior to skin testing. One-tenth milliliter injections were given intradermally using 26-gauge intradermal bevel needles. After a designated time interval, a 5% solution of pontamine sky blue dye in saline was injected intravenously to accentuate the zone of the erythematous response. Dye was given at a dose of 0.8 ml/kg. The diameter of the blue zones, which corresponds to the areas of erythema, were measured in millimeters after 2 and 24 hours. Intensity of induration was graded on a scale of 1 + to 4+, and the zones of delayed bluing were approximately the same as the area of induration.

<u>Histology of Skin Tests</u>. One-tenth ml intradermal injections of the following samples were injected into adult rabbits: 1) a Sephadex G-100 chromatographed fraction of culture filtrate which had both rapid and delayed skin permeability activity, 2) a 1 µg/ml solution of purified cholera toxin as a positive control, and 3) sterile Brain Heart Infusion broth (Difco) as a negative control. Eighteen hours after the last skin injection, the rabbit was anesthesized (Ketamine HCl, Parke-Davis) and a 4 mm punch biopsy was taken from each injection site as well as from normal skin. All biopsies included the hypodermis, dermis, and epidermis. Specimens were fixed in 0.05 M phosphate buffered formalin (pH 7.0). Fixed specimens were sent to the University of Texas Medical Branch pathology lab for sectioning and histological staining with hematoxylin and eosin. Stained sections were analyzed microscopically for pathological signs such as edema and cellular infiltration.

<u>Electrophoresis</u>. Standard 7.5% polyacrylamide gels were loaded with 100 microliter samples and electrophoresed at pH 8.3. One set of gels was stained with Coomassie Blue R 250, and the stained gels were scanned at 590 nm. A duplicate set of unstained gels was sliced into 5 mm segments, which were placed in separate tubes with 0.5 ml of 0.01 M Tris-HCl buffer, pH 7.5. The elutions from each gel slice were tested in rabbit skin for the presence of the delayed PF.

<u>Enzyme Treatment</u>. Enzyme solutions containing 1 mg/ml were made of the following: pronase (Calbiochem), protease (Sigma), trypsin (Sigma), pepsin (Sigma), carboxy-peptidase (Sigma), and lysozyme (Fulka HG, SC). The toxin source was a pool of active Sephadex G-100 chromatographed

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fractions from S. typhimurium 986 culture filtrate. Mixtures of 0.15 ml
of toxin plus 0.15 ml of each enzyme solution (150 mg) were incubated at
37° C for 3 hours follo   by overnight refrigeration at 4°C. A control
consisting of toxin plus saline was included. Before skin testing 3.75
mg of soybean trypsin inhibitor was added per milliliter of the trypsin-
toxin sample (8). Rabbits were observed for both rapid and delayed skin
permeability reactions.

pH Stability. The Salmonella toxin sample chosen was a pool
of biologically active G-100 chromatography fractions (in Tris-HCl
buffer) containing both rapid and delayed permeability activity. A
predetermined volume of acid or base solution was added to each tube.
The desired pH values were 2, 4, 6, 7, 8, and 10. Tris-HCl buffer (pH
7.5) controls (0.5 ml) were also adjusted to the various pH values. The
pH-adjusted toxin samples and controls were incubated at 37° C for 4
hours and then stored overnight at 4° C. The pH was not readjusted to 7.0
before skin testing. Biological activity was determined by skin testing
of all samples, and both rapid and delayed permeability activities were
recorded.

Heat Stability. A 10 mg/ml solution of Salmonella LPS (Difco)
and a chromatographed sample containing Salmonella delayed PF were
boiled in a water bath for one hour. The samples and unheated controls
were then skin tested in rabbits to compare the stability of any rapid
and/or delayed bluing and induration activity in both the exo- and
endotoxin samples.

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Electrofocusing. Sephadex G-75 was prepared according to the instructions given in the LKB application note No. 198. A 5% (w/v) gel slurry was made with the specially dried G-75 gel and a pH 3.5 to 8.5 ampholine solution and a 3 ml sample of crude culture filtrate was added to the slurry. After focusing for 18 hrs at 10°C, the gel bed was sliced into 30 equal fractions, and the gel from each section transferred to individual syringe (5cc) columns. One gel volume of Tris-HCl buffer, pH 7.5, was added to each column and the eluates were assayed for the presence of toxin by the rabbit skin test model.

Antibody Neutralization of Skin Permeability Factors. Monospecific antiserum to cholera toxin was prepared by intradermal injections of 10 μg of purified cholera toxin (5,6) mixed with Freund's complete adjuvant. Antiserum to somatic antigens of Salmonella groups A-I was a commercial preparation (Difco). Studies were conducted to compare the ability of these antisera to neutralize the Salmonella delayed PF and a commercial Salmonella LPS preparation (Difco 5 ng/ml). Each serum was mixed 1:2 with an aliquot of either the Salmonella delayed PF or Salmonella LPS. The mixtures were incubated for 1 hour at 37°C prior to assay for biological activity using the rabbit skin permeability model.

Ganglioside Inactivation of Skin Permeability. Six different ganglioside preparations were used, five of which were kindly provided by Dr. W.E. van Heyningen (Sir William Dunn School of Pathology, Oxford, England) and one of which was a commercial preparation (Supelco) of purified G_{M1} ganglioside. The gangliosides sent by Dr. van Heyningen

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were: SGGnSSLC (G_{T1})*, GGnSLC (G_{M1}), SGGnSLC (G_{D1a}), mixed gangliosides,
mixed "slow" gangliosides $G_{T1}+G_{D1b}+G_{D1a}$. Each preparation was rehydrated
with water and dilutions were made yielding final concentrations of 100
µg/ml and were mixed 1:2 with a biologically active Sephadex G-100
column fraction of Salmonella delayed PF. These mixtures were incubated at
37°C for 3 hours prior to assay by skin testing in rabbits.

Live Cell Challenge of Procholeragenoid Immunized Rabbits.

Five adult rabbits were each immunized with three 200 microgram subcutaneous
doses of procholeragenoid, prepared by heating highly purified choleragen
(5,6) at 60°C for 5 minutes (7). The fluid accumulation responses of
these rabbits were compared with these of 5 normal rabbits when subjected
to intestinal loop challenge with live Salmonella typhimurium strain 986
ranging from 10^3-10^8 organisms/ml two weeks after the last injection.
Challenge procedures were the same as those described for V. cholerae
studies (9).

Results

The skin reaction produced by the Salmonella delayed PF is
illustrated in Figure 1. The site of injection is markedly indurated
with a corresponding zone of erythema. Adjacent sites which were injected
with purified cholera toxin are indistinguishable from the Salmonella
toxin reaction sites. The sites of firm induration with erythema appear

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* = sialic acid, G = galactose, Gn = N-acetylgalactosamine, L = lactose,
C = ceramide

to become maximal in 24 hr and often begin to subside by 36-48 hr. This characteristic is useful in distinguishing the heat labile delayed PF from a heat stable endotoxin reaction, which reaches a maximal response by 48 hr. The latter induration response has been observed with void volume column fractions of Salmonella culture filtrates in which large amounts of endotoxin have been released. In addition, the erythematous response to endotoxin is much more intense with some sites becoming necrotic; whereas, the delayed PF reaction sites appear normal after induration has receded.

Comparisons of histological sections were made of skin biopsies from sites injected with purified cholera toxin (1 μg/ml), Salmonella G-100 chromatographed filtrate with delayed PF, or sterile BHI culture medium. Sections of normal skin and skin sites injected with uninoculated sterile culture media were unremarkable at both 2 hours and 18 hours (Figure 2). The fraction of chromatographed Salmonella filtrate and cholera toxin which had been injected 18 hours prior to fixation produced diffuse edema fluid and PMN infiltration of the epidermis and dermis (Figures 3 and 4). Histologically, the delayed (18 hour) responses of Salmonella PF and cholera toxin were remarkably similar.

Figure 5 shows the typical elution profile of Salmonella typhimurium delayed PF on a column of Sephadex G-100 (5 X 150 cm). The preparation containing the toxin consisted of a pool of concentrated crude culture filtrates which had been previously adsorbed onto DEAE-Sephadex at pH 8 and eluted at pH 6 prior to chromatography. Alternate

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fractions were assayed by the rabbit skin test method. The vertical bars in Figure 5 indicate a bimodal curve of delayed PF activity, with the   first peak also containing rapid PF activity. Figure 6 shows the calibration curve for this G-100 column with the region exhibiting delayed PF activity indicated between the dotted lines. The center of this wide range of molecular sizes is approximately 90,000 daltons. Similar molecular size estimates have been made using Sephadex G-150.

Since the heat labile skin permeability activity routinely appears to be spread over a broad range of elution volumes, three pools containing the active region were made as indicated in Figure 5. When samples of the three pools were electrophoresed on 7.5% polyacrylamide gels at pH 8.3 and stained for protein with Coomassie blue, the three pools yielded bands of protein migrating in three sequential areas of the gels as expected (Figure 7). Although the charge of the molecules affects the migration pattern in these gels, the data confirms that the Sephadex G-100 column allowed separation of a variety of molecular species on the basis of size. When identical, unstained gels were sliced and the gel slice eluates were skin tested, it was observed that the delayed PF activity entered into the same area of the gel, irregardless of which pool was examined (Figure 8). This suggested that the Salmonella delayed PF was more homogeneous in molecular size than the broad elution pattern on Sephadex G-100 would indicate.

Figure 9 illustrates an early attempt to determine the isoelectric point of the Salmonella delayed PF. A sample of crude culture filtrate concentrate was electrofocused with a broad range ampholyte solution (pH

3.5 - 3.5). The skin test assay of electrofocused gel elutions showed that induration activity had distributed along the pH gradient and focused around an isoelectric point of pH 4.8. Although not shown here, an isoelectric point of pH 4.8 was also obtained with a crude polymyxin extract (10) of Salmonella typhimurium 986 cells. More recently, we have determined that the isoelectric point of the delayed PF isolated from the Salmonella typhimurium 2000 strain was pH 4.3. These determinations should prove useful in future purification efforts for the delayed PF.

In order to determine the sensitivity of the Salmonella toxins to extremes in pH, aliquots of a sample containing both the rapid and delayed PF's, and aliquots of a purified cholera toxin solution (1 ug/ml) were adjusted to pH's ranging from 2 to 10. As shown in Figure 10, the skin test assay revealed that the Salmonella rapid PF was stable to all pH's tested, while the Salmonella delayed PF was significantly inactivated at pH 10 and below pH 4. Cholera toxin was also confirmed to be labile at acid and alkaline extremes in pH.

Table 1 summarizes the effect of several enzymes on a chromatographed sample of culture filtrate containing both the rapid and delayed PF from S. typhimurium 986. The data indicate that neither the delayed nor the rapid permeability factor was altered in intensity or in size of reaction by any of the enzymes tested. Similarly, each of the enzyme treated preparations was subjected to 7.5% polyacrylamide electrophoresis. The toxin eluted from the gel slices did not appear to be significantly altered in its electrophoretic migration pattern by any of the enzymes examined.

We have observed that LPS preparations ≥ 4mg/ml elicit an induration response within 18 hours which reaches a maximum within 48 hours. Since the latter skin activity could be confused with the heat labile delayed PF response, comparisons of heat sensitivities were studied. Table 2 compares the heat stability characteristics of lipopoly-saccharide from S. typhimurium and E. coli with the Salmonella delayed PF. Heating the LPS preparations at 100°C for 1 hour had no effect on their ability to cause induration and bluing; whereas the Salmonella delayed PF was completely destroyed.

Since the Salmonella delayed PF appeared to be similar to cholera toxin, an experiment was designed to determine if the tissue receptor might be the same for the two toxins. Figure 11 shows that both cholera toxin and Salmonella delayed PF will compete with [125]I - labeled cholera toxin for binding sites on guinea pig intestinal homogenates. Based on this evidence, purified ganglioside preparations were examined for their ability to bind the toxins. Table 3 summarizes the ability of selected gangliosides to inactivate Salmonella delayed PF and purified cholera toxin. Preincubation of the toxins with the ganglioside preparations resulted in inactivation of both the Salmonella delayed PF and purified cholera toxin only by those preparations containing GM1 ganglioside. Thus, the two toxins appear to be remarkably similar in their affinity for ganglioside receptors.

We have previously reported that the Salmonella delayed PF is neutralized by monospecific antiserum to purified cholera toxin (3). A comparative study was subsequently conducted between Salmonella LPS and

Salmonella delayed PF. Table 4 shows that _S. typhimurium_ LPS is not neutralized by cholera antitoxin or by polyvalent _Salmonella_ antiserum. The _Salmonella_ delayed PF was confirmed to be neutralized by cholera antitoxin but not by the _Salmonella_ agglutinating serum. The data provides still another difference between _Salmonella_ LPS and _Salmonella_ delayed PF.

No data presented has indicated that the _Salmonella_ delayed PF is an enterotoxin, and all preparations injected into ligated intestinal loops of rabbits have failed to elicit fluid accumulation. However, live cell challenge of intestinal loops of rabbits immunized with procholeragenoid (Figure 12.) revealed significant protection against fluid accumulation. The protection observed was almost complete even at the highest challenge dose tested. Confirmation of this heterologous protection was achieved recently even when the rabbits were challenged with _S. typhimurium_ strain 2000, another clinical isolate shown to produce the delayed PF.

Discussion

In the course of this study, a clinical isolate of _Salmonella typhimurium_ strain 986 was shown to release two exotoxic factors into the growth medium. Both factors induced vascular permeability changes upon intradermal injection into rabbit skin. One factor produced a reaction rapid in onset and of short duration (rapid PF), while the other (delayed PF) resulted in a gradual reaction peaking in intensity by 18-24 hrs. The _Salmonella_ delayed PF was studied and compared with

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the enterotoxin produced by <u>Vibrio cholerae</u>. A significant degree of relatedness was shown between the two toxins which was manifest in physical and antigenic properties. Although not presented in detail, a second clinical isolate of <u>S. typhimurium</u> designated strain 2000, has been found to produce both rapid and delayed PF's. The latter delayed PF exhibits all of the properties described for the delayed PF produced by <u>S. typhimurium</u> strain 986.

The rabbit skin test model has been used as a screening procedure for samples containing enterotoxic activity (2). The rationale was that the skin permeability factor assay for cholera toxin and <u>E. coli</u> LT is 100-fold more sensitive than the gastrointestinal models (11). Although cell-free preparations of <u>Salmonella</u> delayed PF have failed to elicit fluid accumulation in rabbit ligated loops, the skin permeability assay has enabled us to compare the <u>Salmonella</u> PF's with purified cholera toxin and to determine some of their biological and physical characteristics.

Histological comparisons made between cholera toxin skin reactions and those produced by the <u>Salmonella</u> delayed PF revealed considerable disruption of the collagen fiber network by fluid accumulation. This observation was expected because of the edematous nature of the skin sites. Analysis of the cellular infiltrate revealed that the predominant cell type was polymorphonuclear leukocytes (PMN). This type of reaction is typical of an inflammatory response, but is inconsistent with a hypersensitivity type reaction.

We have not yet been able to satisfactorily define the factor responsible for rapid PF activity. It appears to be a non-neutralizable,

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heat-stable moiety resistant to extremes in pH and to enzymatic degradation. Although the data is not shown, the rapid PF may be endotoxin, or at least associated with endotoxin, since it was observed that rapid permeability alterations could also be observed with high doses (10 mg/ml) of LPS-(Difco) which invariably developed into an indurated delayed response which became markedly erythematous and often necrotic. Rapid PF development is independent of the evolution of the heat labile delayed PF induration response, and necrosis is not associated with either of the recently described Salmonella PF activities.

The delayed PF of Salmonella has been characterized in more detail than the rapid PF because of its remarkable similarity to the heat-labile enterotoxins of V. cholerae and E. coli. All three heat labile toxins are inactivated at 56°C, and they are also susceptible to inactivation at an acid pH. As one might expect for toxins which exert their pathological effect in the intestinal tract, all three toxins are reported to be relatively resistant to enzymatic degradation compared to other protein molecules of similar molecular size.

The Salmonella delayed PF was shown to be a negatively charged molecule at physiological pH (7.0) and possesses an isoelectric point of approximately 4.8. The isoelectric point for cholera toxin was reported to be pH 6.6 by Finkelstein (4) and pH (6.7) by Delaney (12)   , while that for E. coli LT was reported to be 6.9 (13). The low isoelectric point of Salmonella delayed PF is one of the few physical characteristics that distinguish it from cholera toxin.

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One of the most interesting and least understood aspects of these studies on the Salmonella PF's is the necessity of partial purification to obtain the factor which elicits the delayed induration response. Any explanation of the phenomenon at this point is purely hypothetical; however, we have proposed that an inhibitory factor is produced in culture concomitantly with the two permeability factors, and that removal of this inhibitor through chromatography allows expression of the delayed PF. Whether or not this inhibitory substance is an artifact produced in vitro or actually plays some role in the pathogenic mechanism is not known.

Many authors recognize the similarity in fluid and electrolyte content of the diarrheic fluid of salmonellosis and enterotoxin-induced intestinal fluid secretions (1,14), but the production of an enterotoxic factor has not been clearly associated. Sakazaki et al. (15) reported enterotoxic activity in cell-free filtrates of Salmonella species, but there was poor correlation of these results with that of live Salmonella pathogenicity. Also, Koupal and Deibel (16) reported a heat-stable membrane-associated enterotoxin produced by S. enteritidis and by S. typhimurium ; however they could find no similarities between their Salmonella toxin and cholera toxin. Giannella et al. (17) observed that adenylate cyclase was stimulated in intestinal tissues of rabbits during fluid production elicited by Salmonella. This observation together with the fluid and electrolyte content and the near-normal protein composition of Salmonella diarrheic stools implicate a toxin as the mediator of the diarrhea associated with salmonellosis.

The observation reported here that rabbits could be protected against _Salmonella_ induced intestinal fluid loss by immunization with procholeragenoid, not only suggests a novel approach to prophylaxis against _Salmonella_ mediated diarrhea, but more importantly it shows an integral role of the _Salmonella_ delayed PF in the pathogenesis of experimental salmonellosis. Since fluid loss from the intestines of procholeragenoid immunized rabbits was essentially prevented when challenged by live _Salmonella_ cells, it must be concluded that the _Salmonella_ delayed PF which shares antigenic determinants with cholera toxin, is responsible for the fluid loss in experimental salmonellosis. This conclusion does not conflict with previous observations that epithelial cell invasion is necessary for fluid loss, but rather provides an explantation for the mechanism by which the pathogen evokes the response. The lack of fluid loss from the small intestine following luminal injection of the _Salmonella_ delayed PF may be the result of low concentrations of toxin injected or may indicate a need for the _Salmonella_ to penetrate the epithelium and deliver the delayed PF to the proper receptor. The latter possibility seems less likely since the delayed PF was observed to bind to the same cell receptor as cholera toxin (GM1 ganglioside).

In the last three years, we have observed that the _Salmonella_ heat stable, rapid PF is routinely produced under a variety cultural conditions by all _Salmonella_ serotypes tested. Thus, its production by _Salmonella_ species appears to be a stable genetic characteristic. In recent months, we have been unable to detect delayed PF production from _S. typhimurium_ strain 986. We have investigated innumerable cultural conditions over a period of several months and have now come to the

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The observation reported here that rabbits could be protected against _Salmonella_ induced intestinal fluid loss by immunization with procholeragenoid, not only suggests a novel approach to prophylaxis against _Salmonella_ mediated diarrhea, but more importantly it shows an integral role of the _Salmonella_ delayed PF in the pathogenesis of experimental salmonellosis. Since fluid loss from the intestines of procholeragenoid immunized rabbits was essentially prevented when challenged by live _Salmonella_ cells, it must be concluded that the _Salmonella_ delayed PF which shares antigenic determinants with cholera toxin, is responsible for the fluid loss in experimental salmonellosis. This conclusion does not conflict with previous observations that epithelial cell invasion is necessary for fluid loss, but rather provides an explantation for the mechanism by which the pathogen evokes the response. The lack of fluid loss from the small intestine following luminal injection of the _Salmonella_ delayed PF may be the result of low concentrations of toxin injected or may indicate a need for the _Salmonella_ to penetrate the epithelium and deliver the delayed PF to the proper receptor. The latter possibility seems less likely since the delayed PF was observed to bind to the same cell receptor as cholera toxin ($GM1$ ganglioside).

In the last three years, we have observed that the _Salmonella_ heat stable, rapid PF is routinely produced under a variety cultural conditions by all _Salmonella_ serotypes tested. Thus, its production by _Salmonella_ species appears to be a stable genetic characteristic. In recent months, we have been unable to detect delayed PF production from _S. typhimurium_ strain 986. We have investigated innumerable cultural conditions over a period of several months and have now come to the

conclusion that S. typhimurium strain 986 no longer possesses the genetic capacity to produce delayed PF. We report here the isolation of a new clinical isolate of Salmonella typhimurium designated strain 2000 which produces a delayed PF which is neutralized by GM1 ganglioside and monospecific cholera antitoxin. The delayed PF from this latest S. typhimurium isolate is destroyed in 5 minutes at $100^{\circ}C$ and migrates on the G-100 column in the same location as that reported for the delayed PF from strain 986 with a tendency to trail out over a broad range of elution volumes. In addition, it has been shown to have approximately the same isoelectric point. Therefore, there is no question that the new isolate elaborates delayed PF with the same physical and antigenic properties of our initial isolate. This finding also confirms that S typhimurium strain 986 was not a genetically unique isolate and has provided much needed encouragement to our research endeavors. We now believe that delayed PF production by Salmonella species is an unstable phenomenon whose genetic basis is unknown. We are continuing to study and purify the delayed PF from S. typhimurium strain 2000 and hope that the role of this toxin and that of the heat stable, rapid PF in the pathogenesis of salmonellosis may soon be elucidated.

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Figure and Table Legends

Figure 1.   Skin response showing the indurated reaction to intradermal injections of Salmonella delayed PF and purified cholera toxin after 24 hours without dye injection. Arrows pointing down indicate cholera toxin reactions while the arrows pointing up indicate the Salmonella delayed PF reactions.

Figure 2.   Hematoxylin and eosin stained histological section of rabbit skin injected 24 hours previously with sterile uninoculated BHI.

Figure 3.   Hematoxylin and eosin stained histological section of rabbit skin injected 24 hours previously with purified cholera toxin.

Figure 4.   Hematoxylin and eosin stained histological section of rabbit skin injected 24 hours previously with partially purified Salmonella delayed PF.

Figure 5.   Elution profile of Salmonella delayed PF culture filtrate concentrate eluted from DEAE Sephadex. The dotted line indicates optical density at 280 nm, while the vertical bars show the location of Salmonella delayed PF activity. Three pools were made as marked by the arrows.

Figure 6.   Calibration curve for the Sephadex G-100 column referred to in Figure 5. The dotted lines indicate the range of elution volumes exhibiting delayed PF induration activity. An arrow marks the peak of induration intensity.

Figure 7.   Optical density scans at 590 nm for polyacrylamide gels of the three pools from the Sephadex G-100 column illustrated in Figure 5.

Figure 8.   Diameters of bluing zone reactions of delayed PF eluted from 0.5 cm slices of unstained polyacrylamide gels identical to those illustrated in Figure 7.

Figure 9.   Isoelectric point analysis of a preparation of crude filtrate containing the Salmonella delayed PF. The solid line indicates the pH gradient while the dashed line shows the optical density at 280 nm. The fractions with delayed PF activity are marked by a vertical bar.

Figure 10.   Sensitivity of the Salmonella rapid PF, delayed PF, and cholera toxin to pH.

Figure 11.   Ability of a partially purified preparation of Salmonella delayed PF and cholera toxin to compete with ^{125}I - cholera toxin for binding sites on guinea pig intestinal mucosal homogenates.

Figure 12.   Protection of rabbits against Salmonella typhimurium mediated fluid loss by immunization with procholeragenoid.

Table 1. Resistance of <u>Salmonella</u> rapid and delayed PF to enzymatic degradation. Rapid and delayed PF activities are indicated by the 2 hour and 24 hour reactions, respectively.

Table 2. Heat stability characteristics of the skin permeability activity elicited by LPS and <u>Salmonella</u> delayed PF.

Table 3. Inactivation of skin permeability activity using ganglioside preparations.

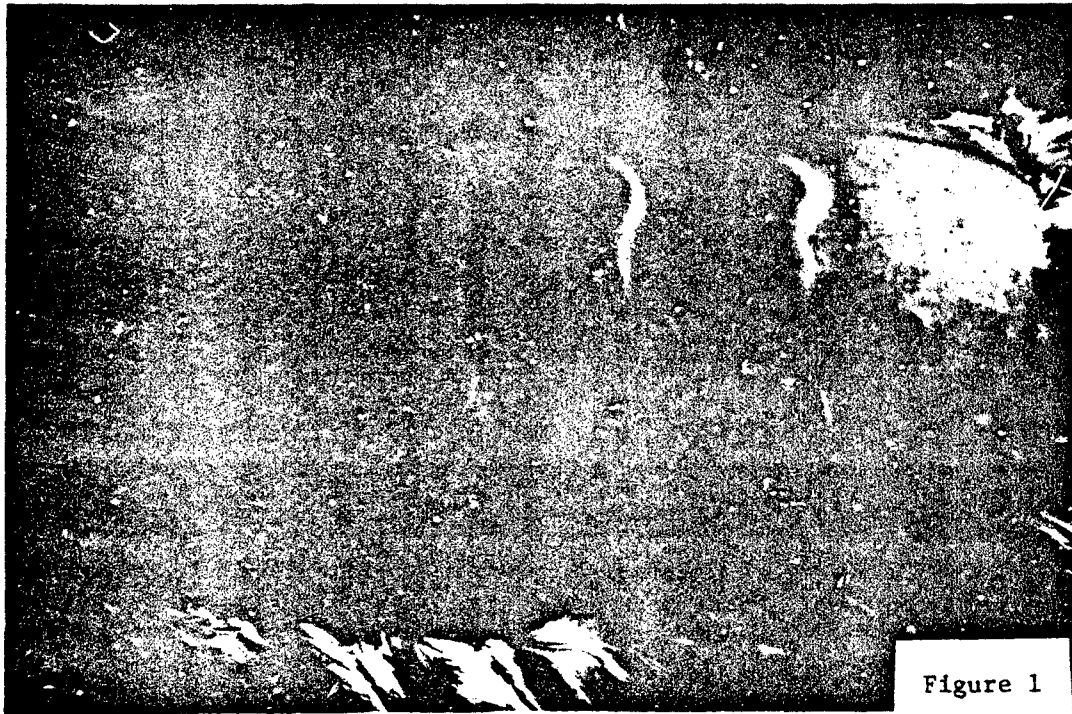Table 4. Antibody neutralization properties of skin permeability activity of <u>Salmonella</u> LPS and delayed PF.

Figure 1

Figure 2
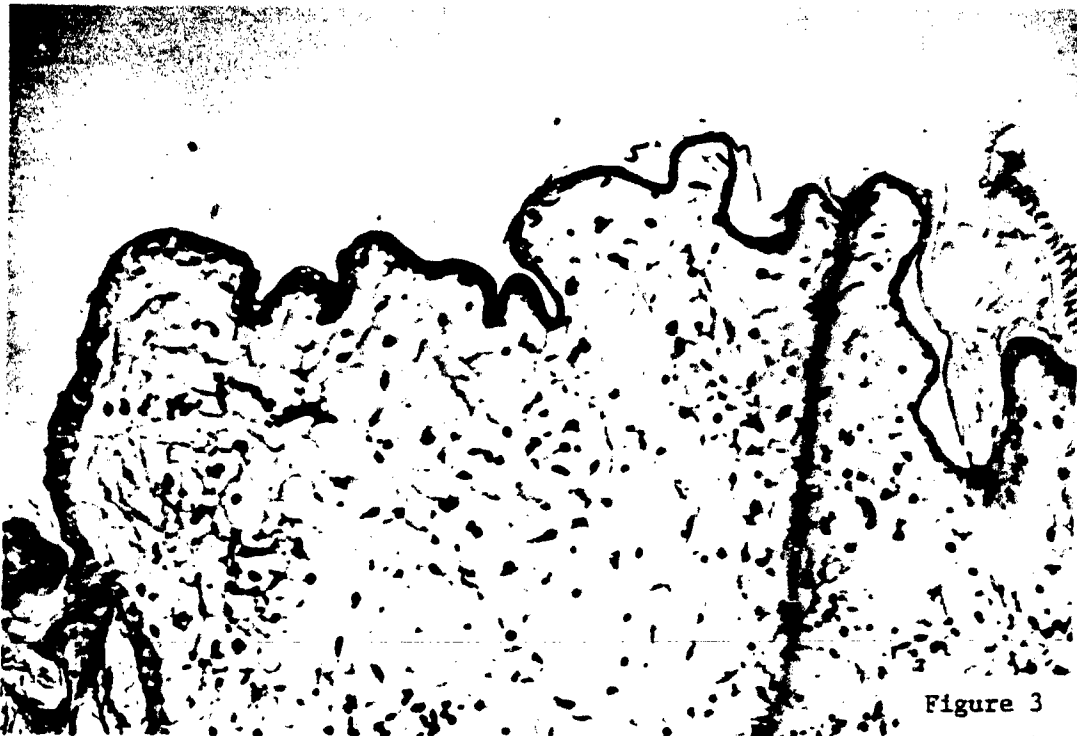
Figure 3

Figure 4
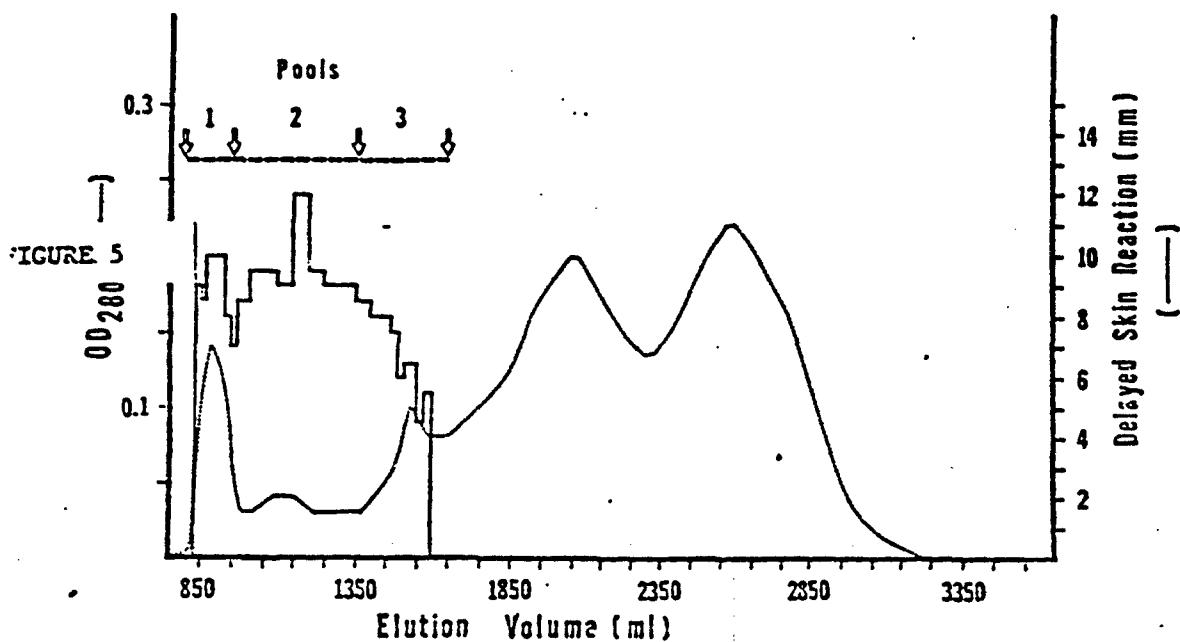
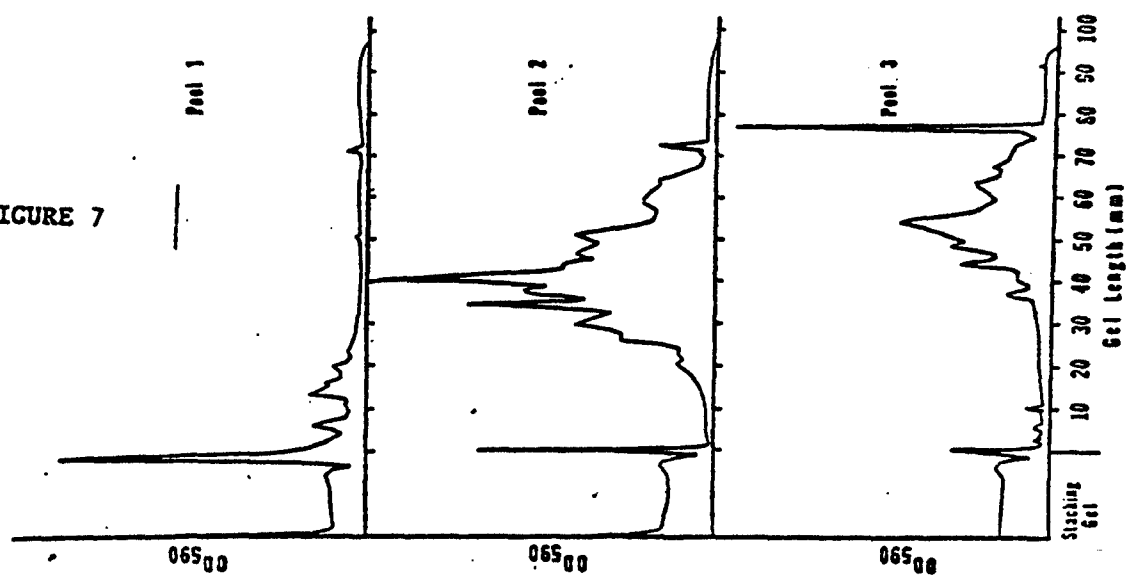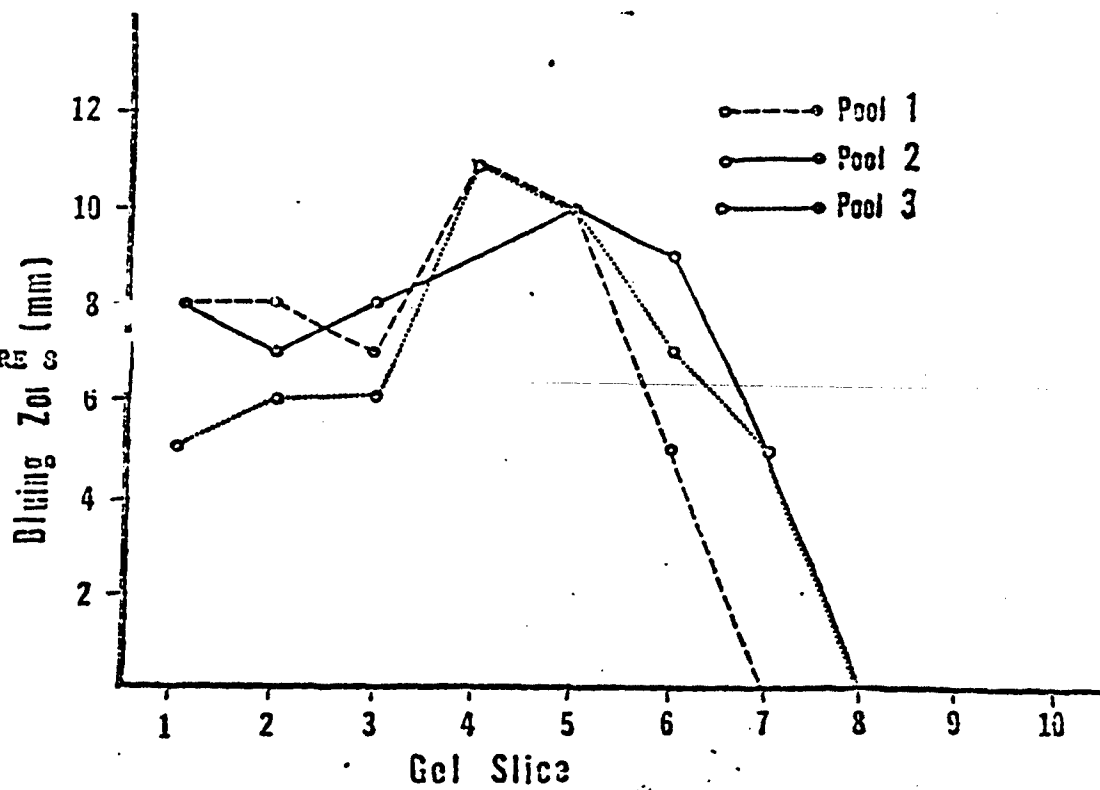FIGURE 5
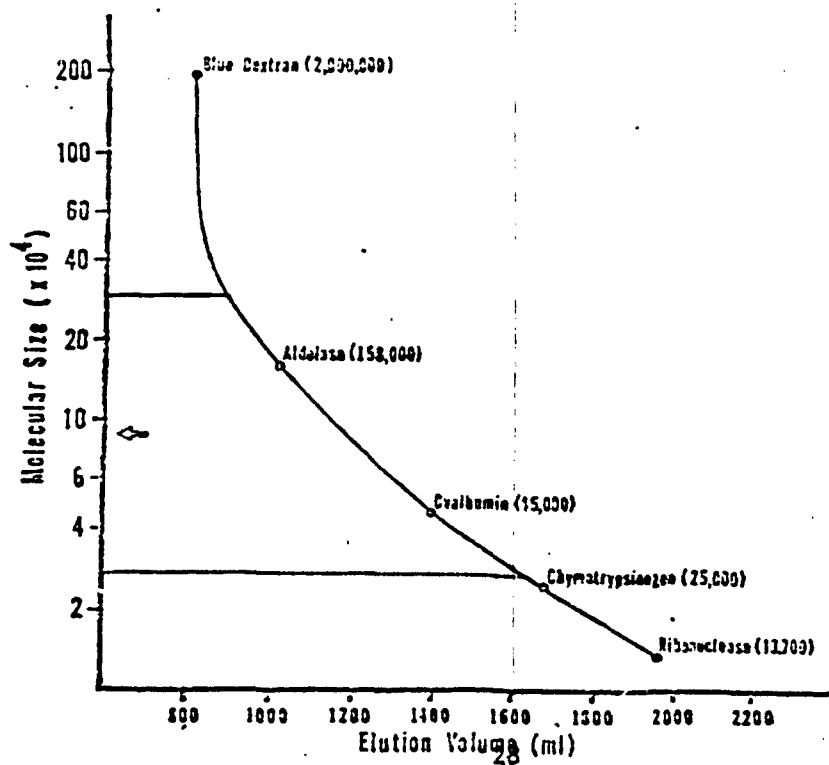
FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 9
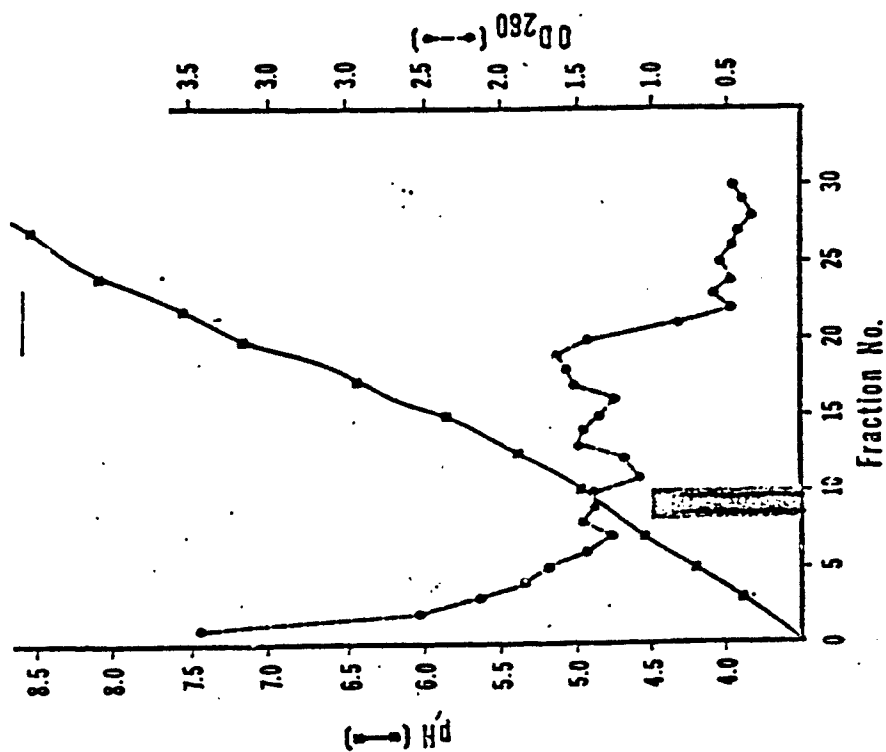
SENSITIVITY of SALMONELLA TOXINS and CHOLERA TOXIN to pH

FIGURE 10

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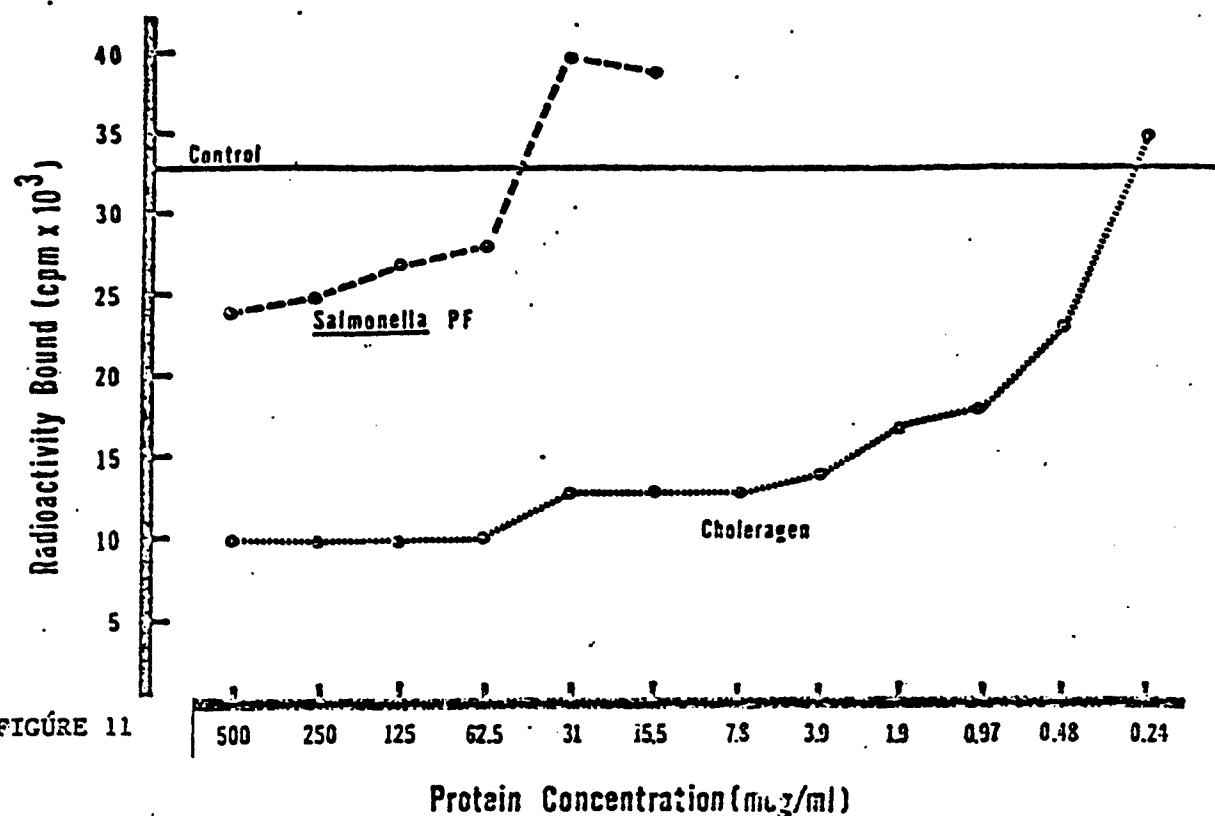
FIGURE 11

Protein Concentration (mɤg/ml)
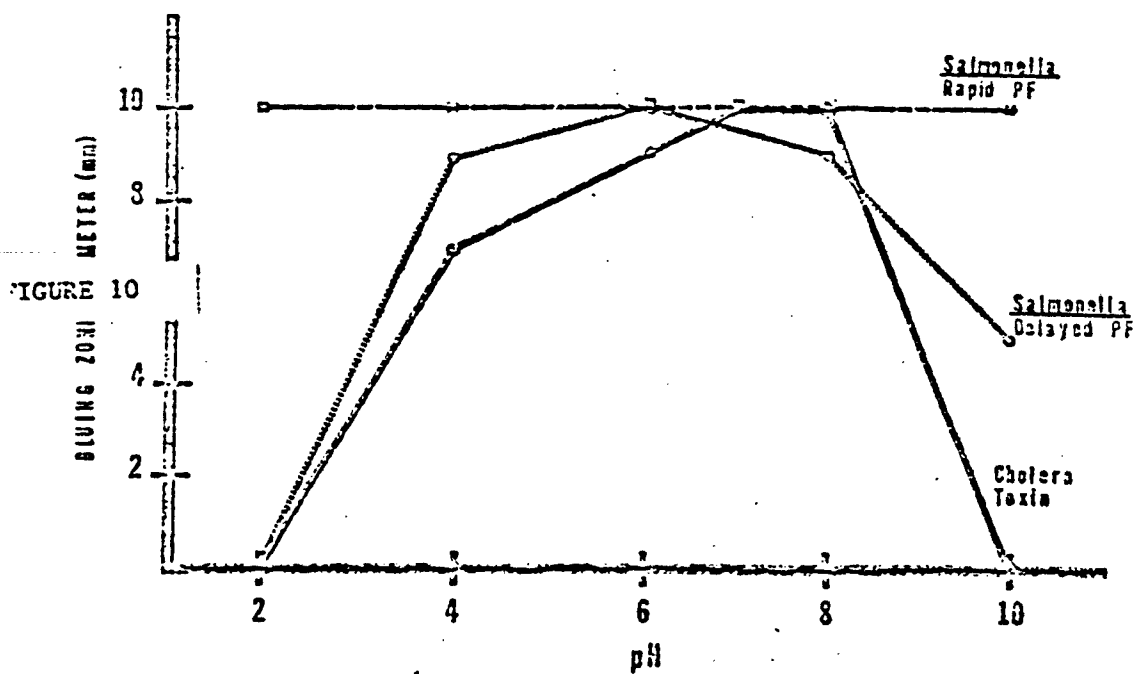
INACTIVATION OF SKIN PERMEABILITY ACTIVITY USING GANGLIOSIDE PREPARATIONS

	GT_1	GD1a	Mixed "slow" without GM_1	Mixed including GM_1	GM_1 Van Heyningen	GM_1 Supelco
Salmonella delayed PF	+	+	+	-	-	-
Cholera toxin	+	+	+	-	-	±

+ indicates an indurated skin response at 18-24 hours;
- indicates no visible response;
± indicates a skin response reduced markedly from that of the control.

TABLE 3

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TABLE 4

NEUTRALIZATION PROPERTIES OF SKIN PERMEABILITY ACTIVITY OF
ENDOTOXIN AND _SALMONELLA_ DELAYED PF

	Polyvalent Salmonella antiserum (Difco)	Cholera antitoxin	Tris Buffer Control
S. typhimurium LPS (Difco) 5 mg/ml	*9 mm/+	8 mm/+	6 mm/+
S. typhimurium delayed PF	8 mm/+	0/0	7 mm/+

* D. meter of bluing zone/intensity of induration at 24 hours

FIGURE 12

FLUID ACCUMULATION (ml/cm)

NONIMMUNIZED CONTROL

IMMUNIZED with PROCHOLERAGENOID
200mcg. 3 injections

LOOP CHALLENGE DOSE
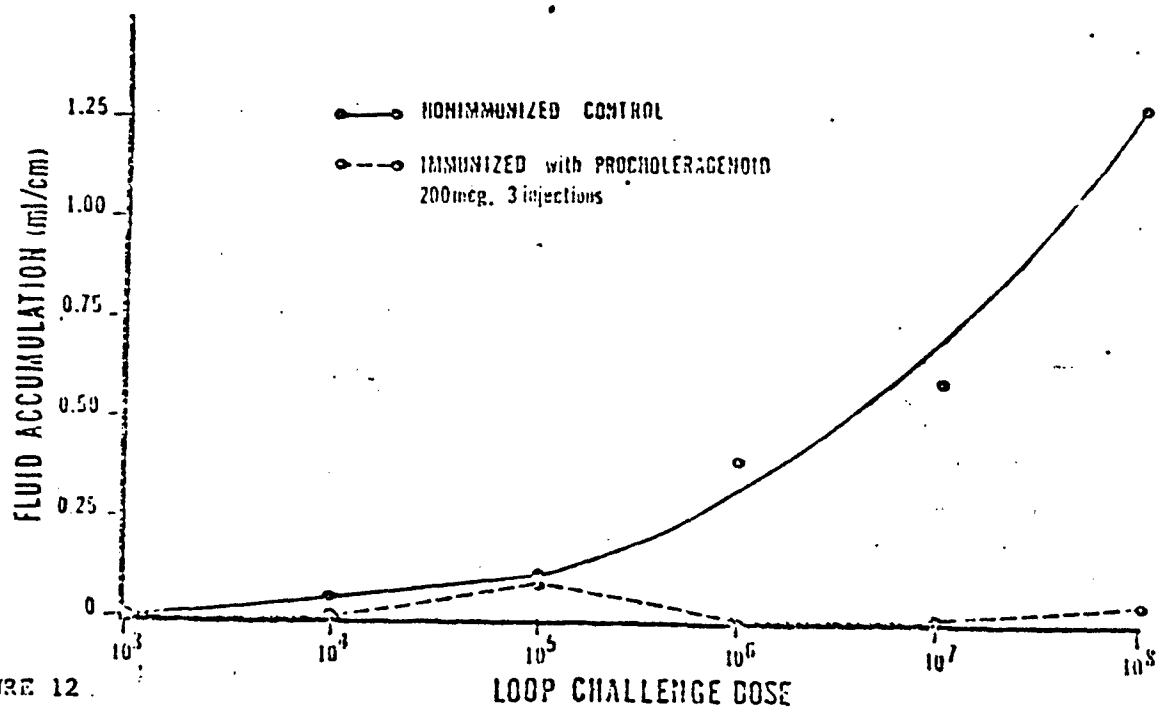
III. Additional Results (not reported in manuscript)

One limitation in the study of the delayed PF has been the requirement
that culture filtrates be chromatographed on Sephadex columns in order to
remove inhibitory factor(s). This procedure was quite time consuming and has
made survey type studies (i.e. cultural conditions and bacterial strains) ex-
ceedingly difficult. In order to assay 10 crude culture filtrate samples, at least
5 days are required using our two fraction collectors. Considering our present
state of knowledge concerning the stability of the delayed PF, we feel uncomfort-
able in comparing preparations treated over extended periods of time. In order to
circumvent this problem, we have been studying various procedures of more rapidly
removing the delayed PF from crude culture filtrates of Salmonella. A variety
of procedures have been tried, but precipitation with sodium metaphosphate has
been yielding excellent results .   We have been able to remove all detect-
able delayed PF activity from fermenter or Fernbach shaken cultures (TSB+1%YE) by
adding sodium metaphosphate (2.55g/1) and lowering the pH to 4.6. The protein
precipitate is collected by filtration and washed with a cold solution of 0.2M
saline (pH 4.6). The ppt is dissolved in phosphate buffered saline (pH 7.2) and
subsequently dialyzed against this buffer. This procedure has allowed us to re-
move the brown culture medium components and inhibiting factor in a single
step, but we have not yet determined the percent recovery of delayed PF using .
this procedure. We are presently using this technique to generate a supply of
partially purified delayed PF. In addition, we are presently attempting to use
the procedure on small volumes of culture filtrates from a variety of culture
media incubated at various temperatures. Despite the fact that the procedure is
a marked improvement over chromatography, the process is still cumbersome.

We have also been using the mouse lymphosarcoma cell culture assay of Murphy
(   ). for detection and quantitation of the Salmonella delayed PF. Our exper-
ience has led us to believe that the assay is not as sensitive as the rabbit skin
test and is particularly susceptible to bacterial contamination. Since the cells
must be incubated for several days with the toxin solution, all samples must be
filter sterilized and kept sterile. Skin test procedures require that samples
initially be filter sterilized, but it is not necessary to keep them sterile.
Frequently, samples that are positive by skin test (firm induration in 18-24 hrs
that is prevented by GM1 ganglioside, cholera antitoxin, and heating at 100°C for
5 minutes) are negative by this cell assay. Due to these disadvantages, we will
be examining other cell assays such as the adrenal cell assay to improve assay
techniques for the delayed PF.

We have been attempting to use standard immunological techniques to detect
and/or quantitate the delayed PF. A large number of concentrated culture filtrates
(10 X-via PM10 ultrafiltration) have been examined by ring tests and Ouchterlony
analysis, using several cholera antitoxic sera (rabbit). The cholera antitoxic
sera yield precipitin bands in both tests, but we have been unable to associate
ring formation in the ring precipitin test with the presence of skin reactive
toxin (delayed PF). Ouchterlony analysis indicated that cholera antitoxic sera
formed 2-3 bands against S. typhimurium strain 986 filtrate concentrates. None
of these formed a reaction of identity with cholera toxin or its A and B subunits.
However, after this work was done, we realized that strain 986 was no longer
producing detectable amounts of delayed PF. When the same studies are repeated
with concentrated filtrates from S. typhimurium strain 2000 (a strain currently
yielding delayed PF), we may be able to determine the antigenic relationship
between Salmonella delayed PF and cholera toxin.

We are not yet sure about the nature of the bands formed between the cholera antitoxic sera and crude Salmonella filtrates. They may be due to experience of the rabbits with Salmonella antigens as a result of infection; however, preimmunization sera did not form the bands.

We have attempted to prepare Salmonella delayed PF antiserum in rabbits, but again the Salmonella preparations which we used for immunization were not active since they came from strain 986. We will repeat these procedures with filtrates from strain 2000, now that we better understand the lability of toxin production, at least by strain 986. Attempts to use radioimmunoassay techniques, which we have used for cholera toxin, have not yet proved successful. We feel that perhaps the proper cross reactive antiserum has not yet been used. Actually the technique may be much more productive when we obtain a monospecific, homologous antiserum.

Finally, we have confirmed our initial observation that immunization of rabbits with procholeragenoid (heated cholera toxin) will protect them against fluid loss from intestinal loop challenge with live Salmonella typhimurium. It should be added that a different S. typhimurium strain was used for challenge of the more recent set of procholeragenoid immunized rabbits. In contrast, we have also immunized rabbits with glutaraldehyde toxoid (Wyeth 20201) and challenged them with S. typhimurium. Interestingly, no protection was achieved using equivalent doses of glutaraldehyde toxoid (200ug, 3 doses). Therefore, it appears that glutaraldehyde treatment may have destroyed cross reactive antigenic determinants, or otherwise interfered with the cross protection. This protection data confirms prior data in which antigenic similarities were observed between Salmonella delayed PF and cholera toxin. In addition, it has reassured us that the Salmonella delayed PF is involved in the pathogenesis of experimental salmonellois.